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Plant PRPP amidotransferase

The present invention relates to the identification of plant PRPP amidotransferase (phosphoribosyl-pyrophosphate amidotransferase, E.C. 2.4.2.14) as novel target for herbicidal active ingredients. The present invention furthermore relates to DNA sequences encoding a polypeptide with PRPP amidotransferase activity. Moreover, the invention relates to the use of a nucleic acid encoding a protein with PRPP amidotransferase activity which originates from plants for generating an assay system for identifying herbicidally active PRPP amidotransferase inhibitors and to plant PRPP amidotransferase identified using this assay system. The invention furthermore relates to the use of the nucleic acid SEQ-ID No. 1 or SEQ-ID No. 3 encoding plant PRPP amidotransferase for the generation of plants with an increased resistance to PRPP amidotransferase inhibitors and for the generation of plants with a modified purine nucleotide content. Moreover, the invention relates to a method of eliminating undesired vegetation, where the plants to be eliminated are treated with a compound which binds specifically to PRPP amidotransferase encoded by a DNA sequence SEQ-ID No 1 or a DNA sequence which hybridizes with this DNA sequence, and inhibits its function.

Plants are capable of synthesizing their cell components from carbon dioxide, water and inorganic salts.

This process is only possible by exploiting biochemical reactions for synthesizing organic substances. Nucleotides are synthesized de novo in plants. Being components of the nucleic acids, they are particularly important. Covalently bound, nucleotides activate carbohydrates for polysaccharide biosynthesis. They furthermore activate head groups for lipid biosynthesis. Nucleotides are involved in virtually all metabolic pathways. Nucleoside triphosphates, especially ATP, drive most of the energy-requiring reactions of the cell. Adenine nucleotides are additionally also found as components in essential factors such as coenzyme A and in nicotinamide and flavin coenzymes, which are involved in a large number of cellular reactions. The coupled hydrolysis of guanosine-5'-triphosphate (GTP) defines a direction of reaction for various cellular processes such as protein translation, assembly of microtubuli, vesicular transport, signal transduction and cell division. Furthermore, nucleotides constitute the starting metabolites for the biosynthesis of

methylxanthines such as caffeine and theobromine in the plant family of the Rubiaceae and Theaceae.

Genes which encode PRPP amidotransferase have been isolated from a variety of organisms.

cDNAs which encode PRPP amidotransferase have been isolated and characterized from various bacterial, animal and vegetable organisms. Plant PRPP amidotransferase cDNAs have been isolated via complementation of E. coli purF mutants and via DNA hybridization techniques from Glycine max, Vigna aconitifolia and from Arabidopsis thaliana (Ito et al., Plant Molecular Biology 26(1994), 529-533; Kim et al., The Plant Journal 7(1995), 77-86). Sequence homology suggests that the encoded enzymes as well as the E. coli PRPP amidotransferase contain 4Fe-4S clusters. The plant PRPP amidotransferase amino acid sequences, which in comparison with E. coli are extended at the N terminus, show similarity to plastid signal sequences.

Several PRPP amidotransferase isoenzymes which are expressed differentially are found in plants. The RNA for Arabidopsis thaliana AtATase1, for example, accumulates preferentially in the roots, while the AtATase2 transcripts are found predominantly in young leaves and flowers (Ito et al., Plant Molecular Biology 26(1994), 529-533). In Vigna aconitifolia, a PRPP amidotransferase RNA accumulates mainly in root nodules and is induced in root tissues by L-Glutamine (Kim et al., The Plant Journal 7(1995), 77-86).

Since plants depend on an effective nucleotide metabolism, it can be assumed that the enzymes which are involved in nucleotide biosynthesis are suitable as target for herbicides. Thus, there have already been described active ingredients which inhibit *de novo* purine biosynthesis in plants. An example which may be mentioned is the natural substance hydanthocidin, which, after phosphorylation *in planta* inhibits adenylosuccinate synthetase (ASS); (Siehl et al., Plant Physiol. 110(1996), 753-758).

Inhibitors for enzymes of purine biosynthesis are, moreover, also known for their pharmacological action in animals and microorganisms: folate analogs inhibit, inter alia, the enzyme GAR transformylase and have an antiproliferative, antiinflammatory and immunosuppressant action. Mycophenolic acid (MPA), an IMP dehydrogenase inhibitor in the GMP synthetic pathway, has an antimicrobial, antiviral and immunosuppressant action (Kitchin et al, Journal of the American Academy of

Dermatology 37(1997), 445-449).

Bacterial PRPP amidotransferase can be inhibited for example by glutamine antagonists such as, for example, azaserine, 6-diazo-5-oxo-L-norleucine (DON) or L-2-amino-4-oxo-5-chloropentanoic acid and by mercaptopurine and thioguanosine. Glutamine antagonists are not specific to PRPP amidotransferase and also affect other purine biosynthesis enzymes, such as formylglycinamidine ribotide synthase. The efficacy of glutamine antagonists on plant PRPP amidotransferase is still to be proven.

It is an object of the present invention to provide proof that PRPP amidotransferase in plants is a suitable herbicidal target, to isolate a complete plant cDNA encoding the enzyme PRPP amidotransferase and functionally express it in bacterial or eukaryotic cells, and to produce an efficient and simple PRPP amidotransferase assay system for carrying out inhibitor-enzyme binding studies.

We have found that this object is achieved by the isolation of genes which encode the plant enzyme PRPP amidotransferase, the generation of PRPP amidotransferase antisense constructs, and the functional expression of PRPP amidotransferase in bacterial or eukaryotic cells.

It is an object of the present invention to isolate full-length cDNAs encoding functional PRPP amidotransferase (E.C.2.4.2.14) from tobacco (*Nicotiana tabacum*).

A first subject-matter of the present invention is a DNA sequence SEQ-ID NO. 1 or SEQ-ID NO. 3 containing the encoding region of a plant PRPP amidotransferase from tobacco, see Example 1.

Another subject-matter of the invention is DNA sequences which are derived from SEQ-ID NO. 1 or SEQ-ID NO. 3 or which hybridize with one of these sequences and which encode a protein which has the biological activity of a PRPP amidotransferase.

Tobacco plants of the line Nicotiana tabacum cv. Samsun NN which carry a PRPP amidotransferase antisense construct have been characterized in greater detail. The plants show different degrees of retarded growth and bleaching of the leaves. The transgenic lines and the progeny of the 1st and 2nd generation showed a reduced growth in soil. Using Northern hybridization, it was detected that the RNA quantity of PRPP amidotransferase was reduced in plants with reduced growth compared with the wild

type. Furthermore, measurement of the enzyme activity detected that the amount of PRPP amidotransferase activity was reduced in the transgenic lines compared with wild-type plants, see Example 7. Growth retardation and the reduction in PRPP amidotransferase activity correlate. This clear connection identifies PRPP amidotransferase for the first time unambiguously as suitable target protein for herbicidal active ingredients.

To be able to find efficient inhibitors of plant PRPP amidotransferase, it is necessary to provide suitable assay systems with which inhibitor/enzyme binding studies can be carried out. To this end, for example, the complete cDNA sequence of tobacco PRPP amidotransferase is cloned into an expression vector (pQE, Qiagen) and overexpressed in E. coli, see Example 2.

Alternatively, however, it is possible to express the expression cassette containing a DNA sequence of SEQ-ID No. 1 or SEQ-ID NO. 3 for example in other bacteria, in yeasts, fungi, algae, plant cells, insect cells or mammalian cells, see Example 4.

The PRPP amidotransferase protein which is expressed with the aid of the expression cassette according to the invention is particularly suitable for finding inhibitors which are specific to PRPP amidotransferase.

To this end, for example, the plant PRPP amidotransferase can be employed in an enzyme assay in which the PRPP amidotransferase activity is determined in the presence and absence of the active ingredient to be tested. A comparison of the two activity determinations allows a qualitative and quantitative statement to be made on the inhibitory behavior of the active ingredient to be tested, see Example 3.

The assay system according to the invention allows a multiplicity of chemicals to be tested rapidly and simply for herbicidal properties. Using this method, substances with a potent action can be selected specifically and reproducibly from amongst a large number of substances, in order that further in-depth tests with which the skilled worker is familiar are carried out subsequently with these substances.

The invention furthermore relates to a method of identifying herbicidally active substances which inhibit the PRPP amidotransferase activity in plants, with the following steps:

a) the generation of transgenic plants, plant tissues or plant cells which comprise an additional DNA sequence encoding an enzyme with PRPP amidotransferase activity and which are capable of overexpressing an enzymatically active PRPP amidotransferase;

b) applying a substance to transgenic plants, plant cells, plant tissue or plant parts and to untransformed plants, plant cells, plant tissue or plant parts;

c) determining the growth or the viability of the transgenic and the untransformed plants, plant cells, plant tissue or plant parts after application of the chemical substance; and

d) comparing the growth or the viability of the transgenic and the untransformed plants, plant cells, plant tissue or plant parts after applying the chemical substance;

where a supression of the growth or the viability of the untransformed plants, plant cells, plant tissue or plant parts, but an absence of potent suppression of the growth or viability of the transgenic plants, plant cells, plant tissue or plant parts, confirms that the substance of b) shows herbicidal activity and inhibits the PRPP amidotransferase enzyme activity in plants.

Another subject-matter of the invention is a method of identifying plant PRPP amidotransferase inhibitors with a potentially herbicidal action by cloning the gene of a plant PRPP amidotransferase, overexpressing it in a suitable expression cassette - for example in insect cells - disrupting the cells and employing the cell extract in an assay system for measuring the enzyme activity in the presence of low-molecular-weight chemicals, either directly or after concentration or isolation of the enzyme PRPP amidotransferase.

Another subject-matter of the invention is compounds with a herbicidal action which can be identified with the above-described assay system.

The invention furthermore relates to a method of eliminating undesired vegetation, where the plants to be eliminated are treated with a compound which binds specifically to plant PRPP amidotransferase and inhibits its function.

Herbicidally active PRPP amidotransferase inhibitors can be employed as defoliants, desiccants, haulm killers and, in particular, as herbicides. Weeds in the widest sense are to be understood as meaning all plants which grow in locations where they are undesired. Whether the active ingredients found with the aid of the assay system according to the invention act as total or selective herbicides depends, inter alia, on the quantity applied.

Herbicidally active PRPP amidotransferase inhibitors can be used, for example, against the following weeds:

Dicotyledonous weeds of the genera:
Sinapis, Lepidium, Galium, Stellaria, Matricaria, Anthemis, Galinsoga, Chenopodium, Urtica, Senecio, Amaranthus, Portulaca, Xanthium, Convolvulus, Ipomoea, Polygonum, Sesbania, Ambrosia, Cirsium, Carduus, Sonchus, Solanum, Rorippa, Rotala, Lindernia, Lamium, Veronica, Abutilon, Emex, Datura, Viola, Galeopsis, Papaver, Centaurea, Trifolium, Ranunculus, Taraxacum.

Monocotyledonous weeds of the genera:
Echinochloa, Setaria, Panicum, Digitaria, Phleum, Poa, Festuca, Eleusine, Brachiaria, Lolium, Bromus, Avena, Cyperus, Sorghum, Agropyron, Cynodon, Monochoria, Fimbristylis, Sagittaria, Eleocharis, Scirpus, Paspalum, Ischaemum, Sphenoclea, Dactyloctenium, Agrostis, Alopecurus, Apera.

Subject-matter of the invention are also expression cassettes whose sequence encodes a tobacco PRPP amidotransferase or its functional equivalent. The nucleic acid sequence can be, for example, a DNA or a cDNA sequence.

In addition, the expression cassettes according to the invention comprise regulatory nucleic acid sequences which govern the expression of the encoding sequence in the host cell. In accordance with a preferred embodiment, an expression cassette according to the invention encompasses upstream, i.e. at the 5′ end of the encoding sequence, a promoter, and downstream, i.e. at the 3′ end, a polyadenylation signal and, if appropriate, other regulatory elements which are operatively linked to the encoding sequence for the PRPP amidotransferase gene, which sequence lies between the promoter and the polyadenylation signal. Operative linkage is to be understood as meaning the sequential arrangement of promoter, encoding sequence, terminator and, if appropriate, other regulatory elements in such a manner that each of the

regulatory elements can function as intended when the encoding sequence is expressed.

An expression cassette according to the invention is generated by fusing a suitable promoter with a suitable PRPP amidotransferase DNA sequence and a polyadenylation signal using customary recombination and cloning techniques as they are described, for example, by T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

Subject-matter of the invention are also functionally equivalent DNA sequences which encode a PRPP amidotransferase gene and which show a sequence homology with the DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3 of 40 to 100%, based on the total length of the DNA sequence.

Preferred subject-matter of the invention are functionally equivalent DNA sequences which encode a PRPP amidotransferase gene and which show a sequence homology with the DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3 of 60 to 100%, based on the total length of the DNA sequence.

Particularly preferred subject-matter of the invention are functionally equivalent DNA sequences which encode a PRPP amidotransferase gene and which show a sequence homology with the DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3 of 80 to 100%, based on the total length of the DNA sequence.

Functionally equivalent sequences which encode a PRPP amidotransferase gene are in accordance with the invention those sequences which retain the desired functions, despite a deviating nucleotide sequence. Functional equivalents thus encompass naturally occurring variants of the sequences described herein, but also artificial nucleotide sequences, for example those which have been obtained by chemical synthesis and which are adapted to suit the codon usage of a plant.

A functional equivalent is also to be understood as meaning in particular natural or artificial mutations of an originally isolated sequence which encodes a PRPP amidotransferase and which continues to show the desired function. Mutations encompass substitutions, additions, deletions, exchanges or insertions of

one or more nucleotide residues. Thus, the present invention for example also extends to those nucleotide sequences which are obtained by modifying this nucleotide sequence. The target of such a modification can be, for example, the further delimitation of the encoding sequence contained therein or else, for example, the introduction of further restriction enzyme cleavage sites.

Functionelle equivalents are also those variants whose function is reduced or increased compared with the starting gene or gene fragment.

In addition, the expression cassette according to the invention can also be employed for the transformation of bacteria, cyanobacteria, yeasts, filamentous fungi and algae, with the purpose of producing sufficient amounts of the enzyme PRPP amidotransferase.

Another subject-matter of the invention is a tobacco protein characterized by the amino acid sequence SEQ-ID NO: 2 or SEQ-ID No. 4 or derivatives or parts of this protein with PRPP amidotransferase activity.

Subject-matter of the invention are also plant proteins with PRPP amidotransferase activity with an amino acid sequence homology to the tobacco PRPP amidotransferase with the SEQ-ID NO: 2 or SEQ-ID NO. 4 of 20 - 100% identity.

Preferred are plant proteins with PRPP amidotransferase activity with an amino acid sequence homology to the tobacco PRPP amidotransferase with the sequences SEQ-ID NO: 2 or SEQ-ID NO. 4 of 50 - 100% identity.

Particularly preferred are plant proteins with PRPP amidotransferase activity with an amino acid sequence homology to the tobacco PRPP amidotransferases with the sequences SEQ-ID NO: 2 or SEQ-ID NO. 4 of 80 - 100% identity.

It was another object of the invention to overexpress the PRPP amidotransferase gene in plants in order to generate plants which tolerate PRPP amidotransferase inhibitors.

Overexpression, in a plant, of the gene sequence SEQ-ID NO. 1 or SEQ-ID NO. 3, which encodes a PRPP amidotransferase, results in an increased resistance to PRPP amidotransferase inhibitors. The transgenic plants generated thus are also subject-matter of the invention.

Expressional efficacy of the recombinantly expressed PRPP amidotransferase gene can be determined, for example, in vitro by shoot-meristem propagation or by a germination test. Moreover, the expression of a PRPP amidotransferase gene which has been altered in terms of type and level, and its effects on the resistance to PRPP amidotransferase inhibitors can be tested in greenhouse experiments using test plants.

Subject-matter of the invention are also transgenic plants, transformed with an expression cassette according to the invention containing the DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3, which have been made tolerant to PRPP amidotransferase inhibitors by additionally expressing the DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3, and transgenic cells, tissues, parts and propagation material of such plants. Especially preferred in this context are transgenic crop plants such as, for example, barley, wheat, rye, maize, soya, rice, cotton, sugar beet, canola, sunflowers, flax, hemp, potatoes, tobacco, tomatoes, oilseed rape, alfalfa, lettuce and the various tree, nut and grapevine species, and also legumes.

A change in the nucleotide content in plants may be useful under various circumstances. For example, nucleotides are added to plant-based baby formulas to achieve a nutrient composition which corresponds to breast milk. Furthermore, an optimized nucleotide content would be helpful when patients are fed by gastric tube. A reduced purine nucleotide content in nutritional plants is relevant for the dietetic diet of patients suffering from gout. Furthermore, nucleotides make and enhance flavors, so that an altered nucleotide content has an effect on the palatability of plants.

Another subject-matter of the invention are thus plants which, following expression of the DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3 in the plant, have a modified purine nucleotide content. It is preferred to increase the content of the purine nucleotides IMP, AMP and/or GMP, or of their di- or trinucleotides ADP, ATP or GDP and GTP.

A plant with a modified purine nucleotide content is generated, for example, by expressing, in the plant, an additional DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3 in sense or antisense orientation. A modified purine nucleotide content means that both plants with an increased purine nucleotide content (in the case of sense orientation) and plants with a reduced guanosine nucleotide content (in the case of sense orientation

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[cosuppression] or antisense orientation) can be generated.

An increased purine nucleotide content means for the purposes of the present invention for example the artificially acquired ability of an increased purine nucleotide biosynthesis rate by functionally overexpressing the PRPP amidotransferase gene in the plant in comparison with the non-recombinant plant for the duration of at least one plant generation.

Another subject-matter of the invention is the use of plant PRPP amidotransferase for altering the methylxanthine concentration in plants.

Particularly preferred are sequences which ensure targeting into the apoplast, into plastids, into the vacuole, into the mitochondrium, into the endoplasmatic reticulum (ER), or which, owing to the absence of suitable operative sequences, ensure that the product remains in the compartment where it is formed, in the cytosol, (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423).

For example, the plant expression cassette can be introduced into the plant transformation vector pBinAR, see Example 5.

A suitable promoter of the expression cassette according to the invention is, in principle, any promoter which is capable of governing the expression of foreign genes in plants. It is preferred to use, in particular, a plant promoter or a promoter derived from a plant virus. Particularly preferred is the cauliflower mosaic virus CaMV 35S promoter (Franck et al., Cell 21(1980), 285-294). This promoter contains different recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the gene which has been introduced (Benfey et al., EMBO J., 8 (1989), 2195-2202).

The expression cassette according to the invention may also comprise a chemically inducible promoter which allows expression of the exogenous PRPP amidotransferase gene in the plant to be governed at a particular point in time. Such promoters which are described in the literature and which can be used are, inter alia, for example the PRP1 promoter (Ward et al., Plant. Mol. Biol. (1993) 22, 361-366), a salicylic acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP 388186), a tetracyclin-inducible promoter (Gatz et al., Plant J. (1992) 2, 397-404), an abscisic acid-inducible promoter (EP0335528) or an ethanol- or cyclohexanone-inducible promoter

(WO 93/21334).

Particularly preferred promoters are furthermore those which ensure expression in tissues or parts of the plant in which the biosynthesis of purines or their precursors takes place. Promoters which ensure leaf-specific expression must be mentioned in particular. Promoters which must be mentioned are the potato cytosolic FBPase or the potato ST-LSI promoter (Stockhaus et al., EMBO J., 8 (1989) 2445-245).

A foreign protein can be expressed stably in the seeds of transgenic tobacco plants to an extent of 0.67% of the total soluble seed protein with the aid of a seed-specific promoter (Fiedler and Conrad, Bio/Technology 10 (1995), 1090-1094). The expression cassette according to the invention can therefore contain, for example, a seed-specific promoter (preferably the phaseolin promoter, the USP promoter or the LEB4 promoter), the LEB4 signal peptide, the gene to be expressed and an ER retention signal.

The inserted nucleotide sequence encoding a PRPP amidotransferase can be produced synthetically or obtained naturally or contain a mixture of synthetic and natural DNA components. In general, synthetic nucleotide sequences are generated with codons which are preferred by plants. These codons which are preferred by plants can be determined from codons with the highest protein frequency expressed in the plant species of the highest interest. When preparing an expression cassette, a variety of DNA fragments may be manipulated in order to obtain a nucleotide sequence which expediently reads in the correct direction and which is equipped with a correct reading frame. Adaptors or linkers can be added to the fragments in order to link the DNA fragments to each other.

Other suitable DNA sequences are artificial DNA sequences as long as they mediate the desired property by increasing the purine nucleotide content in the plant by overexpressing the PRPP amidotransferase gene in crop plants, as described above by way of example. Such artificial DNA sequences can be determined for example by backtranslating of proteins which have PRPP amidotransferase activity and which have been constructed by means of molecular modeling, or they can be determined by in vitro selection. Especially suitable are encoding DNA sequences which have been obtained by backtranslating a polypeptide sequence in accordance with the host-plant-specific codon usage. The specific codon usage can be determined readily by a skilled worker familiar with methods of plant genetics by means of

computer evaluations of other, known genes of the plant to be transformed.

Other suitable equivalent nucleic acid sequences according to the invention which must be mentioned are sequences which encode fusion proteins, the component of the fusion protein being a plant PRPP amidotransferase polypeptide or a functionally equivalent part thereof. The second part of the fusion protein can be, for example, another polypeptide with enzymatic activity or an antigenic polypeptide sequence, with the aid of which detection of PRPP amidotransferase expression is possible (for example myc-tag or his-tag). However, it is preferably a regulatory protein sequence such as, for example, a signal or transit peptide, which leads the PRPP amidotransferase protein to the desired site of action.

The promoter and terminator regions according to the invention should expediently be provided, in the direction of transcription, with a linker or polylinker containing one or more restriction sites for insertion of this sequence. As a rule, the linker has 1 to 10, in most cases 1 to 8, preferably 2 to 6, restriction sites. In general, the linker within the regulatory regions has a size less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter according to the invention may be native, or homologous, or else foreign, or heterologous, to the host plant. The expression cassette according to the invention comprises, in the 5'-3' direction of transcription, the promoter according to the invention, any sequence and a region for transcriptional termination. Various termination regions can be exchanged for each other as desired.

Manipulations which provide suitable restriction cleavage sites or which eliminate the excess DNA or restriction cleavage sites may also be employed. *In vitro* mutagenesis, primer repair, restriction or ligation may be used in cases where insertions, deletions or substitutions such as, for example, transitions and transversions, are suitable. Complementary ends of the fragments may be provided for ligation in the case of suitable manipulations such as, for example, restriction, chewing-back or filling overhangs for blunt ends.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which correspond essentially to *Agrobacterium tumefaciens* T-DNA polyadenylation signals, in particular those of the gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACH5 (Gielen et al., EMBO J., 3 (1984),

835), or functional equivalents.

To transform a host plant with a DNA encoding PRPP amidotransferase, an expression cassette according to the invention is incorporated, as insertion, into a recombinant vector whose vector DNA contains additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described, inter alia, in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press, Chapters 6/7, 71-119).

The transfer of foreign genes into the genome of a plant is termed transformation. It exploits the above-described methods for transforming and regenerating plants from plant tissues or plant cells for transient or stable transformation. Suitable methods are the protoplast transformation by polyethylene-glycol-induced DNA uptake, the biolistic method using the gene gun, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and agrobacterium-mediated gene transfer. The abovementioned methods are described by, for example, B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993), 128-143, and Potrykus Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225. The construct to be expressed is preferably cloned into a vector which is suitable for the transformation of *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711).

Agrobacteria transformed with an expression cassette according to the invention can equally be used in a known manner for transforming plants, in particular crop plants such as cereals, maize, soya, rice, cotton, sugar beet, canola, sunflowers, flax, hemp, potatoes, tobacco, tomatoes, oilseed rape, alfalfa, lettuce and the various tree, nut and grapevine species, and also legumes, for example by bathing wounded leaves or leaf sections in an agrobacterial suspension and subsequently growing them in suitable media.

The purine biosynthesis site is generally the leaf tissue, so that leaf-specific expression of the PRPP amidotransferase gene is meaningful. However, it is obvious that the purine biosynthesis need not be limited to the leaf tissue, but may also take place in all other remaining parts of the plant in a tissue-specific fashion, for example in fatty seeds.

In addition, constitutive expression of the exogenous PRPP amidotransferase gene is advantageous. On the other hand, inducible expression may also be desirable.

Using the recombination and cloning techniques cited above, the expression cassettes according to the invention can be cloned into suitable vectors which allow them to be multiplied, for example in E. coli. Suitable cloning vectors are, inter alia, pBR332, pUC series, M13mp series and pACYC184. Especially suitable are binary vectors which are capable of replication both in E. coli and in agrobacteria.

Another subject-matter of the invention relates to the use of an expression cassette according to the invention for transforming plants, plant cells, plant tissues or parts of plants. The preferred purpose of the use is to increase the PRPP amidotransferase content in the plant.

Depending on the choice of the promoter, expression may take place specifically in the leaves, in the seeds or in other parts of the plant. Such transgenic plants and their propagation material and their plant cells, tissue or parts are another subject of the present invention.

The invention will now be illustrated by the examples which follow, without being limited thereto.

Examples

Recombinant methods on which the use examples are based:

General cloning methods

Cloning methods such as restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linking DNA fragments, transformation of Escherichia coli cells, growing bacteria and the sequence analysis of recombinant DNA were carried out as described by Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6).

Sequence analysis of recombinant DNA

Recombinant DNA molecules were sequenced using an ABI laser fluorescence DNA sequencer, following the method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA, 74(1977), 5463-5467). Fragments resulting from a polymerase chain reaction were

sequenced and checked to avoid polymerase errors in constructs to be expressed.

Analysis of total RNA from plant tissues

Total RNA from plant tissues was isolated as described by Logemann et al.(Anal. Biochem. 163(1987), 21). For the analysis, in each case 20 µg of RNA were separated in a formaldehyde-containing 1.5% agarose gel and transferred to nylon membranes (Hybond, Amersham). Specific transcripts were detected as described by Amasino (Anal. Biochem. 152(1986), 304). The DNA fragments employed as probe were radiolabeled with a Random Primed DNA Labeling Kit (Roche, Mannheim) and hybridized by standard methods (see Hybond instructions, Amersham). Hybridization signals were visualized by autoradiography with the aid of Kodak X-OMAT AR films.

Unless otherwise specified, the chemicals used were analytical grade and obtained from Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen). Solutions were made with a refined, pyrogen-free water, termed H_2O hereinbelow, from a Milli-Q water refining system (Millipore, Eschborn). Restriction endonucleases, DNA-modifying enzymes and molecular biologic kits were obtained from AGS (Heidelberg), Amersham (Braunschweig), Biometra (Göttingen), Roche (Mannheim), Genomed (Bad Oeynnhausen), New England Biolabs (Schwalbach/Taunus), Novagen (Madison, Wisconsin, USA), Perkin-Elmer (Weiterstadt), Pharmacia (Freiburg), Qiagen (Hilden) and Stratagene (Heidelberg). Unless otherwise specified, they were used in accordance with the manufacturer's instructions.

The bacterial strains used hereinbelow (E. coli, XL-1 Blue) were obtained from Stratagene. E. coli AT 2465 was obtained from the coli genetic stock center (Yale University, New Haven). The agrobacterial strain used for transforming plants (Agrobacterium tumefaciens, C58C1 with plasmid pGV2260 or pGV3850kan) was described by Deblaere et al. (Nucl. Acids Res. 13 (1985), 4777). Alternatively, it is also possible to use the agrobacterial strain LBA4404 (Clontech) or other suitable strains. Vectors which can be used for cloning are pUC19 (Yanish-Perron, Gene 33(1985), 103-119), pBluescript SK- (Stratagene), pGEM-T (Promega), pZerO (Invitrogen), pBin19 (Bevan et al., Nucl. Acids Res. 12(1984), 8711-8720) and pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990), 221-230).

Example 1

Isolation of cDNAs encoding a functional tobacco PRPP amidotransferase.

To isolate PRPP—amidotransferase-encoding cDNAs from Nicotiana tabacum, an *Arabidopsis* PRPP amidotransferase-encoding cDNA clone (AtATase1; Ito et al., Plant Molecular Biology 26(1994), 529-533; GenBank Accession number D28868) was used as template for generating a hybridization probe by means of PCR.

The reaction mixtures contained approx. 1 ng/µl template DNA, 0.5 µM of the oligonucleotide 5'-cgc tct aga act agt gga tc-3' and 5'-tcg agg tcg acg gta tc-3', 200 µM deoxy nucleotide (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C, 1.5 mM $MgCl_2$) and 0.02 U/µl Taq polymerase (Perkin Elmer).

The amplification conditions were set as follows:

Annealing temperature:	50°C, 1 min
Denaturation temperature:	94°C, 1 min
Elongation temperature:	72°C, 2 min
Number of cycles:	30

The resulting 1.9 kb fragment was used for a heterologous screening of a Nicotiana tabacum var. SR-1 (Stratagene) cDNA library. 3.0×10^5 lambda phages of the cDNA library were plated onto agar plates with E. coli XL1-blue as bacterial strain. The phage DNA was transferred to nitrocellulose filters (Gelman Sciences) by means of standard methods (Sambrook et al. (1989), Cold Spring Harbor Laboratory PressL ISBN 0-87969-309-6) and fixed on the filters. The hybridization probe used was the above-described PCR fragment, which was radiolabelled with the aid of the "Multiprime DNA labeling systems" (Amersham Buchler) in the presence of α-^{32}P-dCTP (specific activity 3000 Ci/mmol) following the manufacturer's instructions. The membranes were hydbridized after prehybridization at 60°C in 3 x SSPE, 0.1% sodium dodecyl sulfate (w/v), 0.02% polyvinylpyrrolidone (w/v), 0.02% Ficoll 400 (w/v) and 50 mg/ml calf thymus DNA for approx. 12 hours. The filters were subsequently washed for 60 minutes in 2 x SSPE, 0.1% sodium dodecyl sulfate (w/v) at 60°C. Positively hybridizing phages were visualized by autoradiography and singled out by means of standard techniques (Sambrook et al. (1989); Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6) and transferred into plasmid (Stratagene).

Following restriction and sequence analysis, two different clones were identified, Ntpur1.1 (clone 7.2) containing the DNA sequence SEQ-ID No. 1 and Ntpur1.2 (clone 9.2) containing the DNA sequence SEQ-ID No. 3, which encode reading frames with homology to *Arabidopsis thaliana* AtATAse1. The amino acid sequences of Ntpur1.1 (SEQ-ID No. 2 - length: 573 amino acids) and Ntpur1.2 (SEQ-ID No. 4 - length: 573 amino acids) show 97% identity, see Table 1. The homology with AtATase1 at amino acid level is 81% in the case of Ntpur1.1 and 85% in the case of Ntpur1.2. The continuous reading frames start with nucleotide base 49 (Ntpur1.1) and 25 (Ntpur1.2) respectively, and are translated into polypeptides 573 amino acids in length.

Table 1
Amino acid comparison Ntpur1.1 x Ntpur1.2:

```
  1 MAATVSTASAAATNKSPLSQPLDKPFCSPSQKLLSLSPKTLPKPYRTLVT  50
    |||||||||||||||| ||||||||||| ||||||||||| |||||||:|
  1 MAATVSTASAAATNKYPLSQPLDKPFCSLSQKLLSLSPKTHPKPYRTLIT  50
             .         .         .         .         .
 51 ASSKNPLNDVVSFKKSADNTLDSYFDDEDKPREECGVVGIYGDSEASRLC 100
    ||||||||||:||||||||||||||||:||||||||||||||||||||||
 51 ASSKNPLNDVISFKKSADNTLDSYFDDDDKPREECGVVGIYGDSEASRLC 100
             .         .         .         .         .
101 YLALHALLHRGQEGAGIVAVNDDVLKSITGVGLVSDVFNESKLDQLPGDM 150
    ||||||| ||||||||||||||||||||||||||||||||||||||||||
101 YLALHALQHRGQEGAGIVAVNDDVLKSITGVGLVSDVFNESKLDQLPGDM 150
             .         .         .         .         .
151 AIGHVWYSTAGSSMLKNVQPFVANYKFGSVGVAHNGNLVNYKLLRGELEE 200
    ||||| |||||||||||||||||.||||||||||||||||||||| ||||
151 AIGHVRYSTAGSSMLKNVQPFVASYKFGSVGVAHNGNLVNYKLLRSELEE 200
             .         .         .         .         .
201 NGSIFNTSSDTEVVLHLIAISKARPFLLRIVEACEKIEGAYSMVFVTEDK 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 NGSIFNTSSDTEVVLHLIAISKARPFLLRIVEACEKIEGAYSMVFVTEDK 250
             .         .         .         .         .
251 LVAVRDPHGFRPLVMGRRSNGAVVFASETCALDLIEATYEREVNPGEVVV 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 LVAVRDPHGFRPLVMGRRSNGAVVFASETCALDLIEATYEREVNPGEVVV 300
             .         .         .         .         .
301 VDKDGVHSIYLMPHPEHKSCIFEHIYFALPNSVVFGRSVYESRRAFGEIL 350
    |||||| || |||||| |||||||||||||||||||||||||||||||||
301 VDKDGVQSICLMPHPERKSCIFEHIYFALPNSVVFGRSVYESRRAFGEIL 350
             .         .         .         .         .
351 ATEAPVECDVGIAVPDSGIVAALGYAAKAGVPFQQGLIRSHYVGRTFIEP 400
    |||||||||| |||||||:|||||||||||||||||||||||||||||||
351 ATEAPVECDVVIAVPDSGVVAALGYAAKAGVPFQQGLIRSHYVGRTFIEP 400
             .         .         .         .         .
401 SQKIRDFGVKLKLSPVRALLEGKRVVVVDDSIVRGTTSSKIVRLLKEAGA 450
    ||||||||||||||||||.|||||||||||||||||||||||||||||||
401 SQKIRDFGVKLKLSPVRAVLEGKRVVVVDDSIVRGTTSSKIVRLLKEAGA 450
             .         .         .         .         .
451 KEVHMRIASPPIIASCYYGVDTPSSDELISNRMSVEEIKEFIGSDSLAFL 500
    ||||||||||||||||||||||||||||||||||||||||||||||||||
451 KEVHMRIASPPIIASCYYGVDTPSSDELISNRMSVEEIKEFIGSDSLAFL 500
             .         .         .         .         .
```

```
501 PMDSLNKLLGNDSKSFCYACFSGNYPVEPTGKVKRIGDFMDDGLSGDMDS 550
    ||||||||||||||||||||||||||||||||||||||||||||||||||
501 PMDSLNKLLGNDSKSFCYACFSGNYPVEPTGKVKRIGDFMDDGLSGDMDS 550
                .         .
551 IDGGWLPGSSRVQKTILNEVRTG 573
    ||||||||||||||||||||||
551 IDGGWLPGSSRVQKTILNEVRTS 573
```

Compared with bacterial and human PRPP amidotransferase sequences, the plant proteins (Ntpur1.1, Ntpur1.2, AtATase1) show an extended N-terminus with a large proportion of basic amino acids (Table 2), which suggests the function of a transit peptide for plastid import (von Heijne et al., Eur. J. Biochem. 180(1989), 535-545).

Table 2
Sequence comparison of Arabidopsis thaliana (AtATase1), Bacillus subtilis (BacSu_purF), Human (pur1_hum) and Nicotiana tabacum (Ntpur1.1), Ntpur1.2) PRPP amidotransferase proteins.

```
           1                                                  50
  AtATase1 ---------- ---------- -------SLN QTILLTPINL SLSSPNPSLN
BacSu_purF ---------- ---------- ---------- ---------- ----------
    Ntpur1 LAPHLLFLLS SFFPPPMAAT VSTASAAATN KSPLSQPLDK PFCSPSQKL.
  Ntpur1-2 --------LS SFFPPPMAAT VSTASAAATN KYPLSQPLDK PFCSLSQKL.
  pur1_hum ---------- ---------- ---------- ---------- ----------
           51                                                100

  AtATase1 LHISLS.FLL PSPLLLLHSS MESPPTSPLL HHPKNNSHAP FDYHNDEDDE
BacSu_purF ---------- ---------- ---------- ---------- ----MLAEIK
    Ntpur1 ..LSLSPKTL PKPYRTLVTA SSKNPLNDVV SFKKSADNTL DSYFDDED..
  Ntpur1-2 ..LSLSPKTH PKPYRTLITA SSKNPLNDVI SFKKSADNTL DSYFDDDD..
  pur1_hum ---------- ---------- ---------- ---------- ----MELEEL
           101                                               150

  AtATase1 KPREECGVVG IYGDPE.... ..ASRLFYLA LHALQHRGQE GAGIVTVSPE
BacSu_purF GLNEECGVFG IWGHEE.... ..APQITYYG LHSLQHRGQE GAGIVATDGE
    Ntpur1 KPREECGVVG IYGDSE.... ..ASRLCYLA LHALLHRGQE GAGIVAVN.D
  Ntpur1-2 KPREECGVVG IYGDSE.... ..ASRLCYLA LHALQHRGQE GAGIVAVN.D
  pur1_hum GIREECGVFG CIASGEWPTQ LDVPHVITLG LVGLQHRGQE SAGIVTSDGS
           151                                               200

  AtATase1 KV..LQTITG VGLVSEVFNE SKLDQL.PGE FAIAHVRYST AGASMLKNVQ
BacSu_purF K...LTAHKG QGLITEVFQN GELSKV.KGK GAIGHVRYAT AGGGGYENVQ
    Ntpur1 DV..LKSITG VGLVSDVFNE SKLDQL.PGD MAIGHVWYST AGSSMLKNVQ
  Ntpur1-2 DV..LKSITG VGLVSDVFNE SKLDQL.PGD MAIGHVRYST AGSSMLKNVQ
  pur1_hum SVPTFKSHKG MGLVNHVFTE DNLKKLYVSN LGIGHTRYAT TGKCELENCQ
           201                                               250

  AtATase1 PFV.AGYRFG SIGVAHNGNL VNYKTLRAML EENGSIFNTS SDTEVVLHLI
BacSu_purF PLLFRSQNNG SLALAHNGNL VNATQLKQQL ENQGSIFQTS SDTEVLAHLI
    Ntpur1 PFV.ANYKFG SVGVAHNGNL VNYKLLRGEL EENGSIFNTS SDTEVVLHLI
  Ntpur1-2 PFV.ASYKFG SVGVAHNGNL VNYKLLRSEL EENGSIFNTS SDTEVVLHLI
  pur1_hum PFVVETLH.G KIAVAHNGEL VNAARLRKKL LRHGIGLSTS SDSEMITQLL
           251                                               300

  AtATase1 AISKAR.... ..PFFMRIID ACEKLQGAYS MVFVTEDKLV AVRDPYGFRP
```

```
BacSu_purF KRSGHF.... ..TLKDQIKN SLSMLKGAYA FLIMTETEMI VALDPNGLRP
    Ntpur1 AISKAR.... ..PFLLRIVE ACEKIEGAYS MVFVTEDKLV AVRDPHGFRP
  Ntpur1-2 AISKAR.... ..PFLLRIVE ACEKIEGAYS MVFVTEDKLV AVRDPHGFRP
  pur1_hum AYTPPQEQDD TPDWVARIKN LMKEAPTAYS LLIMHRDVIY AVRDPYGNRP
           301                                              350

  AtATase1 LVMGR..... .........R SNGAVVFASE TCALDLIEAT YEREVYPGEV
BacSu_purF LSIGM..... .........M GD.AYVVASE TCAFDVVGAT YLREVEPGEM
    Ntpur1 LVMGR..... .........R SNGAVVFASE TCALDLIEAT YEREVNPGEV
  Ntpur1-2 LVMGR..... .........R SNGAVVFASE TCALDLIEAT YEREVNPGEV
  pur1_hum LCIGRLIPVS DINDKEKKTS ETEGWVVSSE SCSFLSIGAR YYREVLPGEI
           351                                              400

  AtATase1 LVVDKDGVKS QCLMPKFEPK Q...CIFEHI YFSLPNSIVF GRSVYESRHV
BacSu_purF LIINDEGMKS ERFSMNINRS I...CSMEYI YFSRPDSNID GINVHSARKN
    Ntpur1 VVVDKDGVHS IYLMPHPEHK S...CIFEHI YFALPNSVVF GRSVYESRRA
  Ntpur1-2 VVVDKDGVQS ICLMPHPERK S...CIFEHI YFALPNSVVF GRSVYESRRA
  pur1_hum VEISRHNVQT LDIISRSEGN PVAFCIFEYV YFARPDSMFE DQMVYTVRYR
           401                                              450

  AtATase1 FGEILATESP VECDVVIAVP DSGVVAALGY AAKSGVPFQQ GLIRSHYVGR
BacSu_purF LGKMLAQESA VEADVVTGVP DSSISAAIGY AEATGIPYEL GLIKNRYVGR
    Ntpur1 FGEILATEAP VECDVGIAVP DSGIVAALGY AAKAGVPFQQ GLIRSHYVGR
  Ntpur1-2 FGEILATEAP VECDVVIAVP DSGVVAALGY AAKAGVPFQQ GLIRSHYVGR
  pur1_hum CGQQLAIEAP VDADLVSTVP ESATPAALAY AGKCGLPYVE VLCKNRYVGR
           451                                              500

  AtATase1 TFIEPSQKIR DFGVKLKLSP VRGVLEGKRV VVVDDSIVRG TTSSKIVRLL
BacSu_purF TFIQPSQALR EQGVRMKLSA VRGVVEGKRV VMVDDSIVRG TTSRRIVTML
    Ntpur1 TFIEPSQKIR DFGVKLKLSP VRALLEGKRV VVVDDSIVRG TTSSKIVRLL
  Ntpur1-2 TFIEPSQKIR DFGVKLKLSP VRAVLEGKRV VVVDDSIVRG TTSSKIVRLL
  pur1_hum TFIQPNMRLR QLGVAKKFGV LSDNFKGKRI VLVDDSIVRG NTISPIIKLL
           501                                              550

  AtATase1 REAGAKEVHM RIASPPIVAS CYYGVDTPSS EELISNRLSV EEINEFIGSD
BacSu_purF REAGATEVHV KISSPPIAHP CFYGIDTSTH EELIASSHSV GEIRQEIGAD
    Ntpur1 KEAGAKEVHM RIASPPIIAS CYYGVDTPSS DELISNRMSV EEIKEFIGSD
  Ntpur1-2 KEAGAKEVHM RIASPPIIAS CYYGVDTPSS DELISNRMSV EEIKEFIGSD
  pur1_hum KESGAKEVHI RVASPPIKYP CFMGINIPTK EELIANKPEF DHLAEYLGAN
           551                                              600

  AtATase1 SLAFLSFDTL KKHL...... .......... .....GK... .DSK.SFCYA
BacSu_purF TLSFLSVEGL LKGI...... .......... .....GRKYD .DSNCGQCLA
    Ntpur1 SLAFLPMDSL NKLL...... .......... .....GN... .DSK.SFCYA
  Ntpur1-2 SLAFLPMDSL NKLL...... .......... .....GN... .DSK.SFCYA
  pur1_hum SVVYLSVEGL VSSVQEGIKF KKQKEKKHDI MIQENGNGLE CFEKSGHCTA
           601                                              650

  AtATase1 CFTGDYPVKP TEVKVKRGGG DFIDDGLVGS FENIEAGWVR ----------
BacSu_purF CFTGKYPTEI YQDTVLPHVK EAVLTK---- ---------- ----------
    Ntpur1 CFSGNYPVEP TG.KVKR.IG DFMDDGLSGD MDSIDGGWLP GSSRVQKTIL
  Ntpur1-2 CFSGNYPVEP TG.KVKR.IG DFMDDGLSGD MDSIDGGWLP GSSRVQKTIL
  pur1_hum CLTGKYPVEL EW-------- ---------- ---------- ----------
           651

  AtATase1 ------
BacSu_purF ------
    Ntpur1 NEVRTG
  Ntpur1-2 NEVRTS
  pur1_hum ------
```

Example 2

Expression of tobacco PRPP amidotransferase in E. coli

The purpose of expressing Ntpur1.2 in E. coli was to prove that the Ntpur1.2-encoded PRPP amidotransferase enzyme was active. To this end, a 1523 bp fragment was amplified in a PCR with Pfu polymerase using the oligonucleotides Jle336: 5'-ttttgctagcgactcgtattttgacg-3' and Jle337: 5'-aaaaagatctcaggttctaacttcat -3' and Ntpur1.2 DNA as template. The DNA fragment generated encodes a PRPP amidotransferase enzyme with is truncated N-terminally by 86 amino acids and no longer contains the transit peptide to be received. This truncated form of PRPP amidotransferase enzyme starts N-terminally with the amino acids MDSYFDDDD. Using the oligonucleotides, an NheI cleavage site and a BglII cleavage site were inserted via which the fragment generated was ligated into the NheI- and BamHI-cleaved expression vector pET11a (Novagen).

For expression, the E. coli strain BL21(DE3)LysS (Novagen) was transformed with the construct pETNtpur1.2 which had thus been generated. Following overnight culture, a day culture was inoculated to OD_{600} = 0.1 and, after an OD_{600} = 0.7 had been reached, induced with 1mM IPTG. A total cell extract was produced by the pressure disruption method ("French press") in 50mM Tris-HCl, pH 7.4; 150mM NaCl. Following SDS polyacrylamide gel electrophoresis, an overexpressed protein of approx. 65 kDa was excised from the gel. To produce antisera, the protein was injected into rabbits (contractor: Eurogentec, Herstal, Belgium).

Example 3

Assay system for measuring the activity of plant PRPP amidotransferase activity

The above-described method for measuring plant PRPP amidotransferase activity by the method of Reynolds et al. (Archives of Biochemistry and Biophysics 229 (1984), 623-631) is not suitable for high-throughput assaying owing to the use of radioactive materials. This is why an alternative assay system with which the plant PRPP amidotransferase activity is detected in the protein extract is detected on the basis of the formation of the reaction product glutamate, based on the method described by Shid and Ishii (Journal of Biological Chemistry 66 (1969), 175-181) for E. coli PRPP amidotransferase. The concentration of

the glutamate which forms is measured by converting it with glutamate dehydrogenase (GluDH) and monitoring APADH formation photometrically at 363 nm.

$$\text{PRPP} + \text{L-glutamine} \xrightarrow{\text{PRAT}} \text{PRA} + \text{L-glutamate} + \text{PP}_i$$

$$\text{L-glutamate} + \text{APAD} + H_2O \xrightarrow{\text{GluDH}} \alpha\text{-oxoglutarate} + \text{APADH} + NH_4^+$$

(PRPP = phosphoribosyl pyrophosphate, PRA = phosphoribosylamine, APAD = 3-acetylpyridineadenin dinucleotide, PRAT = PRPP amidotransferase)

To this end, the reaction batch (see below) is incubated at 37°C for up to 60 minutes and the reaction was quenched by incubation at 95°C for 5 minutes.

Reaction batch:

375 µl	100 mM	Tris/HCl buffer pH 8.0
75 µl	100 mM	$MgCl_2$
75 µl	30 mM	phosphoribosyl pyrophosphate
75 µl	100 mM	L-glutamine
50 µl		H_2O
100 µl		protein extract
750 µl		

The glutamate formed was detected in the detection batch (see below) by measuring the increase in APADH photometrically at 363 nm following addition of glutamate dehydrogenase.

Detection batch:

375 µl	100 mM	Tris/HCl buffer pH 8.0
75 µl	500 mM	KCl
125 µl		H_2O
75 µl	3 mM	APAD
100 µl		of the reaction batch
750 µl		

Start of the detection reaction with 2 µl (approx. 4 units) glutamate dehydrogenase (Sigma).

The assay system lends itself in particular for measuring PRPP amidotransferase activity from plant material and in expression extracts, for example from baculovirus-infected insect cells.

Example 4

Functional expression of tobacco PRPP amidotransferase in insect cells

The back-to-back expression system from GibcoBRL was employed for expressing Ntpur1.1 in baculovirus-infected insect cells. To this end, Ntpur1.1 was employed in a PCR. The reaction mixtures contained approximately 1 ng/µl Ntpur1.1 DNA, 0.5 µM of the oligonucleotides 5'-tat agg atc cat gga ctc cta ttt tga cg-3' and 5'-atg aat tct agc tgg ttc taa ctt c-3', 200 µM deoxynucleotides (Pharmacia), 0.04 U/µl Pfu polymerase (Stratagene) and buffer conditions were set following the manufacturer's instructions.

The amplification conditions were set as follows:

Step 1:

Denaturation temperature:	95°C, 0.5 min
Annealing temperature:	40°C, 0.5 min
Elongation temperature:	72°C, 2 min
Number of cycles for Step 1:	2

Step 2:

Denaturation temperature:	95°C, 0.5 min
Annealing temperature:	50°C, 0.5 min
Elongation temperature:	72°C, 3 min
Number of cycles for Step 2:	25

The PCR product was ligated into the StuI-cut vector pFastBac1 (GibcoBRL). The correct orientation of the insert was ensured by control digest with KpnI. The resulting transfer vector pFastBacNtpur1.2 was used following the manufacturer's instructions for generating recombinant baculoviruses by means of Sf21 insect cells (Invitrogen). Sf21 insect cells were infected with the recombinant baculovirus (BvNtpur1.2). After 2-4 days, the cells were harvested by centrifugation. A protein of approx. 54kDa, which corresponds to the expected size of PRPP amidotransferase, was identified in the total extract by SDS polyacrylamide gel electrophoresis. A total cell extract was prepared by the pressure disruption method ("French press") in extraction buffer (100 mM HEPES pH 8.0; 2.5 mM EDTA; 10% glycerol; 20 mM DTE; 0.2 mM PEFA block) and, after being freed from salt over a PD10 column (Pharmacia), used for measuring PRPP amidotransferase activity in the assay described (see Example 3).

Example 5

Generation of plant transformation vectors

To generate binary vectors for plant transformation, clone Ntpur1.1 was cleaved with SmaI and EcoRV, and a fragment comprising 1482 bp was isolated and ligated into the SmaI-cleaved vector pBinAR (Höfgen and Willmitzer, Plant Science 66(1990), 221-230). The antisense and sense constructs thus obtained were termed pBinAR-Ntpur1A and pBinAR-Ntpur1, respectively; see Figure 1.

Example 6

Generation of transgenic tobacco plants

Plasmid pBinAR-Ntpur1A and pBinAR-Ntpur1 were transformed into Agrobacterium tumefaciens C58C1:pGV2260 (Deblaere et al., Nucl. Acids. Res. 13(1984), 4777-4788). To transform tobacco plants (Nicotiana tabacum cv. Samsun NN), a 1:50 dilution of an overnight culture of a positively transformed agrobacterial colony in Murashige-Skoog medium (Murashige and Skoog, Physiol. Plant. 15(1962), 473) supplemented with 2% sucrose (2MS medium) was used. Leaf disks of sterile plants (in each case approx. 1 cm^2) were incubated in a Petri dish for 5-10 minutes with a 1:50 agrobacterial dilution. This was followed by 2 days' incubation in the dark at 25°C on 2MS medium with 0.8% Bacto agar. Cultivation was continued after 2 days and at 16 hours light/8 hours dark and continued in a weekly rhythm on MS medium with 500 mg/l claforan (cefotaxime-sodium), 50 mg/l kanamycin, 1 mg/l benzylaminopurine (BAP), 0.2 mg/l naphthylacetic acid and 1.6 g/l glucose. Growing shoots were transferred to MS medium supplemented with 2% sucrose, 250 mg/l claforan and 0.8% Bacto agar.

Regenerated shoots were obtained on 2MS medium with kanamycin and claforan, transferred into soil after rooting, and, after cultivation for two weeks in a controlled-environment cabinet in a 16-hour light/8-hour dark rhythm at 60% atmospheric humidity, analyzed for PRPP amidotransferase expression and activity and for altered metabolite contents and phenotypic growth characteristics. Altered nucleotide contents can be determined for example following the method of von Stitt et al., FEBS Letters 145(1982), 217-222.

Example 7

Analysis of transgenic plants

Transgenic plants which were transformed with the construct with pBinAR-Ntpur1 are characterized by a growth which is reduced by different degrees and by large-scale bleaching of the leaves in comparison with untransformed control plants (Fig. 2). RNA analysis by the Northern blot technique showed a reduced amount of Ntpur1.1-RNA in transgenic lines with the above-described phenotype (Fig. 3). These effects were also observed in subsequent generations of the transgenic lines.

To test the correlation with growth reduction, PRPP amidotransferase activity in the transgenic lines was measured and compared with that in untransformed controls. To this end, in each case approx. 30 g of leaves from plants approximately 20 cm in height were homogenized with 50 ml of extraction buffer at +4°C.

Extraction buffer:

100 mM	HEPES pH 8,0
2.5 mM	EDTA
10%	glycerol
20 mM	DTE
0,2 mM	PEFA block (40mM)

The disruption extract was filtered through Miracloth (Calbiochem, Bad Soden) and spun at 16,000 rpm in a Sorval centrifuge. The resulting supernatent was precipitated with ammonium sulfate at 4°C. The 30% - 60% fraction was solubilized in an extraction buffer and freed from salt by means of a PD-10 column (Pharmacia, Sweden). The extract thus obtained is stable for at least 24 hours and can be stored over a prolonged period at -20°C after addition of glycerol (end concentration 50%). The extract can be employed directly in the activity determination. Compared to wild-type plants, the PRPP amidotransferase activity in the transgenic lines was markedly reduced, see Fig. 4. Fig. 4A shows the PRPP amidotransferase activity based on the protein quantity. Fig. 4B shows the PRPP amidotransferase activity based on the fresh weight.

These data establish a direct connection between reduced PRPP amidotransferase activity and reduced growth of the tobacco plants and thus identify PRPP amidotransferase for the first time as suitable target protein for herbicidal active ingredients.

Example 8

Search for PRPP amidotransferase activity inhibitors

The in-vitro assay described in Example 3 can be used together with high-throughput methods for searching for PRPP amidotransferase activity inhibitors. To this end, the PRPP amidotransferase activity can be prepared from plant tissue, see Example 7. Alternatively, a plant PRPP amidotransferase can be expressed in E. coli, insect cells or in another suitable expression system. Known PRPP amidotransferase inhibitors such as glutamine antagonists were identified in this manner.

Example 9

Analysis of the adenine and guanine nucleotide contents in transgenic plants.

Leaf material (in each case 5 disks of 6 mm diameter) was harvested from wild-type plants and transgenic plants transformed with the construct pBinAR-Ntpur1 and the subsequent generation (lines 3.1, 3.2, 3.9, 25.1 and 38.8) and frozen immediately in liquid nitrogen. TCA extracts were subsequently prepared by standard methods and employed for the determination of the nucleotide contents.

In the transgenic plants, with the exception of line 38.8, AMP is reduced greatly in the green regions of the leaf and less in the yellow regions of the leaf compared to the wild type (WT) (see Fig. 5).

No changes compared to the wild type were observed for the guanosine nucleotide GTP, GDP and GMP.

SEQUENCE LISTING

<110> BASF Aktiengesellschaft

<120> Plant PRPP amidotransferase

<130> NAE991125

<140>
<141>

<160> 4

<170> PatentIn Vers. 2.0

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<220>
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Met Ala Ala
1

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Thr Val Ser Thr Ala Ser Ala Ala Ala Thr Asn Lys Ser Pro Leu Ser
5 10 15

cag ccc ctc gac aaa ccc ttt tgc tcc cca tct caa aag ctc tta tct 153
Gln Pro Leu Asp Lys Pro Phe Cys Ser Pro Ser Gln Lys Leu Leu Ser
20 25 30 35

tta tcc cct aaa acc ctc cca aaa ccc tat aga act ctc gtc acc gca 201
Leu Ser Pro Lys Thr Leu Pro Lys Pro Tyr Arg Thr Leu Val Thr Ala
40 45 50

tct tcc aaa aac ccc tta aac gac gtc gtt tcg ttt aag aaa tca gct 249
Ser Ser Lys Asn Pro Leu Asn Asp Val Val Ser Phe Lys Lys Ser Ala
55 60 65

gac aat aca ttg gac tcg tat ttt gac gat gaa gac aaa ccc cgt gaa 297
Asp Asn Thr Leu Asp Ser Tyr Phe Asp Asp Glu Asp Lys Pro Arg Glu
70 75 80

gag tgt ggc gtt gtg ggc atc tat ggc gac tca gaa gct tca cgc ctt 345
Glu Cys Gly Val Val Gly Ile Tyr Gly Asp Ser Glu Ala Ser Arg Leu
85 90 95

tgc tat tta gca ctt cac gcg ctt cta cac cgt ggc caa gaa ggc gcc 393
Cys Tyr Leu Ala Leu His Ala Leu Leu His Arg Gly Gln Glu Gly Ala
100 105 110 115

ggc att gtc gcc gtt aac gac gac gtt ctt aag tca att aca ggt gtt 441
Gly Ile Val Ala Val Asn Asp Asp Val Leu Lys Ser Ile Thr Gly Val
120 125 130

ggg tta gta tcc gac gtg ttc aat gag tca aag ctt gac caa ctc cct 489
Gly Leu Val Ser Asp Val Phe Asn Glu Ser Lys Leu Asp Gln Leu Pro
135 140 145

ggt gac atg gca att ggc cac gtc tgg tac tct act gct ggc tct tct 537
Gly Asp Met Ala Ile Gly His Val Trp Tyr Ser Thr Ala Gly Ser Ser
150 155 160

atg tta aaa aat gtt cag cct ttt gtt gct aat tat aaa ttt ggg tca 585
Met Leu Lys Asn Val Gln Pro Phe Val Ala Asn Tyr Lys Phe Gly Ser
165 170 175

gtt ggt gtt gcc cat aat ggt aat tta gtg aat tat aag tta ctg cgt 633
Val Gly Val Ala His Asn Gly Asn Leu Val Asn Tyr Lys Leu Leu Arg
180 185 190 195

ggt gaa cta gaa gag aat ggg tca att ttt aat acg agt tct gat act 681
Gly Glu Leu Glu Glu Asn Gly Ser Ile Phe Asn Thr Ser Ser Asp Thr
200 205 210

gaa gtg gta ctt cac ctt att gct ata tcg aaa gct agg cct ttt tta 729
Glu Val Val Leu His Leu Ile Ala Ile Ser Lys Ala Arg Pro Phe Leu
215 220 225

ttg agg att gtt gag gct tgt gaa aaa att gaa ggt gct tat tct atg 777
Leu Arg Ile Val Glu Ala Cys Glu Lys Ile Glu Gly Ala Tyr Ser Met
230 235 240

gtg ttt gtt act gag gat aag ttg gtt gcc gta agg gat cct cat ggg 825
Val Phe Val Thr Glu Asp Lys Leu Val Ala Val Arg Asp Pro His Gly
245 250 255

ttt agg cca ttg gtt atg ggt agg aga agt aat ggt gct gtt gtt ttt 873
Phe Arg Pro Leu Val Met Gly Arg Arg Ser Asn Gly Ala Val Val Phe
260 265 270 275

gcg tcg gag acg tgt gct ttg gat ttg att gag gct act tat gag agg 921
Ala Ser Glu Thr Cys Ala Leu Asp Leu Ile Glu Ala Thr Tyr Glu Arg
280 285 290

gag gtg aat cct ggt gag gtt gtt gtt gtg gat aaa gat ggg gtc cat 969
Glu Val Asn Pro Gly Glu Val Val Val Val Asp Lys Asp Gly Val His
295 300 305

tct att tat ttg atg cct cat ccc gag cat aaa tct tgt atc ttt gag 1017
Ser Ile Tyr Leu Met Pro His Pro Glu His Lys Ser Cys Ile Phe Glu
310 315 320

cat att tac ttt gct ctg cct aat tcg gtc gtg ttt ggg agg tct gtg 1065
His Ile Tyr Phe Ala Leu Pro Asn Ser Val Val Phe Gly Arg Ser Val
325 330 335

tac gag tct agg cgt gct ttt gga gag att ctt gcg act gaa gct ccc 1113
Tyr Glu Ser Arg Arg Ala Phe Gly Glu Ile Leu Ala Thr Glu Ala Pro
340 345 350 355

gta gaa tgt gat gtt ggg ata gca gtt cct gat tcg ggt atc gtg gct 1161
Val Glu Cys Asp Val Gly Ile Ala Val Pro Asp Ser Gly Ile Val Ala
360 365 370

gcg ctc ggt tat gct gct aaa gcg ggg gta ccg ttt caa caa ggt ttg 1209
Ala Leu Gly Tyr Ala Ala Lys Ala Gly Val Pro Phe Gln Gln Gly Leu
375 380 385

ata agg tcg cat tat gtt ggt agg aca ttt atc gag ccg tcg cag aag 1257
Ile Arg Ser His Tyr Val Gly Arg Thr Phe Ile Glu Pro Ser Gln Lys
390 395 400

ata agg gat ttc ggg gtg aag ctt aag ttg tca cca gtt agg gca tta 1305
Ile Arg Asp Phe Gly Val Lys Leu Lys Leu Ser Pro Val Arg Ala Leu
405 410 415

ttg gag ggg aaa agg gtt gtg gtc gtg gac gat tca atc gtt aga ggg 1353
Leu Glu Gly Lys Arg Val Val Val Val Asp Asp Ser Ile Val Arg Gly
420 425 430 435

acg acc tcg tcc aag att gtg agg ttg ttg aag gag gcg ggt gcg aaa 1401
Thr Thr Ser Ser Lys Ile Val Arg Leu Leu Lys Glu Ala Gly Ala Lys
440 445 450

gag gtt cat atg agg att gca agc cca cca att ata gct tct tgt tat 1449
Glu Val His Met Arg Ile Ala Ser Pro Pro Ile Ile Ala Ser Cys Tyr
455 460 465

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tat gga gtg gat act cct agt tca gat gag ctg ata tca aat agg atg    1497
Tyr Gly Val Asp Thr Pro Ser Ser Asp Glu Leu Ile Ser Asn Arg Met
        470                 475                 480

agt gtg gag gag att aag gag ttc att gga tcg gat tcg ctt gct ttt    1545
Ser Val Glu Glu Ile Lys Glu Phe Ile Gly Ser Asp Ser Leu Ala Phe
    485                 490                 495

ctg cca atg gat agc ttg aat aag ttg tta ggc aat gat tct aaa agc    1593
Leu Pro Met Asp Ser Leu Asn Lys Leu Leu Gly Asn Asp Ser Lys Ser
500                 505                 510                     515

ttt tgc tat gct tgc ttt tcg ggc aat tac ccg gtc gag ccg acg ggt    1641
Phe Cys Tyr Ala Cys Phe Ser Gly Asn Tyr Pro Val Glu Pro Thr Gly
                520                 525                 530

aag gtt aaa agg att ggg gat ttc atg gat gat gga tta agt gga gat    1689
Lys Val Lys Arg Ile Gly Asp Phe Met Asp Asp Gly Leu Ser Gly Asp
            535                 540                 545

atg gat tcc att gat ggt ggt tgg cta cca gga agt agt agg gtt caa    1737
Met Asp Ser Ile Asp Gly Gly Trp Leu Pro Gly Ser Ser Arg Val Gln
        550                 555                 560

aag act atc ttg aat gaa gtt aga acc ggc taaactttct tttccatgtt      1787
Lys Thr Ile Leu Asn Glu Val Arg Thr Gly
    565                 570

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agtctctttt tctaaaaaaa aaaaaaaaaa aa                               1879
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<213> Nicotiana tabacum

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Leu Leu Ser Leu Ser Pro Lys Thr Leu Pro Lys Pro Tyr Arg Thr Leu
        35                  40                  45
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Val Thr Ala Ser Ser Lys Asn Pro Leu Asn Asp Val Val Ser Phe Lys
50 55 60

Lys Ser Ala Asp Asn Thr Leu Asp Ser Tyr Phe Asp Asp Glu Asp Lys
65 70 75 80

Pro Arg Glu Glu Cys Gly Val Val Gly Ile Tyr Gly Asp Ser Glu Ala
85 90 95

Ser Arg Leu Cys Tyr Leu Ala Leu His Ala Leu Leu His Arg Gly Gln
100 105 110

Glu Gly Ala Gly Ile Val Ala Val Asn Asp Asp Val Leu Lys Ser Ile
115 120 125

Thr Gly Val Gly Leu Val Ser Asp Val Phe Asn Glu Ser Lys Leu Asp
130 135 140

Gln Leu Pro Gly Asp Met Ala Ile Gly His Val Trp Tyr Ser Thr Ala
145 150 155 160

Gly Ser Ser Met Leu Lys Asn Val Gln Pro Phe Val Ala Asn Tyr Lys
165 170 175

Phe Gly Ser Val Gly Val Ala His Asn Gly Asn Leu Val Asn Tyr Lys
180 185 190

Leu Leu Arg Gly Glu Leu Glu Glu Asn Gly Ser Ile Phe Asn Thr Ser
195 200 205

Ser Asp Thr Glu Val Val Leu His Leu Ile Ala Ile Ser Lys Ala Arg
210 215 220

Pro Phe Leu Leu Arg Ile Val Glu Ala Cys Glu Lys Ile Glu Gly Ala
225 230 235 240

Tyr Ser Met Val Phe Val Thr Glu Asp Lys Leu Val Ala Val Arg Asp
245 250 255

Pro His Gly Phe Arg Pro Leu Val Met Gly Arg Arg Ser Asn Gly Ala
260 265 270

Val Val Phe Ala Ser Glu Thr Cys Ala Leu Asp Leu Ile Glu Ala Thr
275 280 285

Tyr Glu Arg Glu Val Asn Pro Gly Glu Val Val Val Val Asp Lys Asp
290 295 300

Gly Val His Ser Ile Tyr Leu Met Pro His Pro Glu His Lys Ser Cys
305   310   315   320

Ile Phe Glu His Ile Tyr Phe Ala Leu Pro Asn Ser Val Val Phe Gly
                325   330   335

Arg Ser Val Tyr Glu Ser Arg Arg Ala Phe Gly Glu Ile Leu Ala Thr
            340   345   350

Glu Ala Pro Val Glu Cys Asp Val Gly Ile Ala Val Pro Asp Ser Gly
        355   360   365

Ile Val Ala Ala Leu Gly Tyr Ala Ala Lys Ala Gly Val Pro Phe Gln
    370   375   380

Gln Gly Leu Ile Arg Ser His Tyr Val Gly Arg Thr Phe Ile Glu Pro
385   390   395   400

Ser Gln Lys Ile Arg Asp Phe Gly Val Lys Leu Lys Leu Ser Pro Val
                405   410   415

Arg Ala Leu Leu Glu Gly Lys Arg Val Val Val Val Asp Asp Ser Ile
            420   425   430

Val Arg Gly Thr Thr Ser Ser Lys Ile Val Arg Leu Leu Lys Glu Ala
        435   440   445

Gly Ala Lys Glu Val His Met Arg Ile Ala Ser Pro Pro Ile Ile Ala
    450   455   460

Ser Cys Tyr Tyr Gly Val Asp Thr Pro Ser Ser Asp Glu Leu Ile Ser
465   470   475   480

Asn Arg Met Ser Val Glu Glu Ile Lys Glu Phe Ile Gly Ser Asp Ser
                485   490   495

Leu Ala Phe Leu Pro Met Asp Ser Leu Asn Lys Leu Leu Gly Asn Asp
            500   505   510

Ser Lys Ser Phe Cys Tyr Ala Cys Phe Ser Gly Asn Tyr Pro Val Glu
        515   520   525

Pro Thr Gly Lys Val Lys Arg Ile Gly Asp Phe Met Asp Asp Gly Leu
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Ser Gly Asp Met Asp Ser Ile Asp Gly Gly Trp Leu Pro Gly Ser Ser
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Arg Val Gln Lys Thr Ile Leu Asn Glu Val Arg Thr Gly
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Ala Ala Ala Thr Asn Lys Tyr Pro Leu Ser Gln Pro Leu Asp Lys Pro
10 15 20 25

ttt tgc tcc cta tct caa aag ctc tta tct tta tcc cct aaa acc cat 147
Phe Cys Ser Leu Ser Gln Lys Leu Leu Ser Leu Ser Pro Lys Thr His
30 35 40

cct aaa ccc tac aga act ctc atc acc gcc tct tcc aaa aac ccc tta 195
Pro Lys Pro Tyr Arg Thr Leu Ile Thr Ala Ser Ser Lys Asn Pro Leu
45 50 55

aac gac gtc att tcg ttt aag aaa tca gct gac aat acc ttg gac tcc 243
Asn Asp Val Ile Ser Phe Lys Lys Ser Ala Asp Asn Thr Leu Asp Ser
60 65 70

tat ttt gac gat gac gat aaa ccc cgt gaa gag tgc ggc gtt gtg ggc 291
Tyr Phe Asp Asp Asp Asp Lys Pro Arg Glu Glu Cys Gly Val Val Gly
75 80 85

atc tat ggc gac tca gaa gct tca cgc ctt tgc tat tta gca ctt cac 339
Ile Tyr Gly Asp Ser Glu Ala Ser Arg Leu Cys Tyr Leu Ala Leu His
90 95 100 105

gcg ctt caa cac cgt ggc caa gaa ggc gcc ggc att gtc gcc gtt aac 387
Ala Leu Gln His Arg Gly Gln Glu Gly Ala Gly Ile Val Ala Val Asn
110 115 120

gac gac gtt ctt aag tca att aca ggt gtt ggg tta gta tcc gac gtg 435
Asp Asp Val Leu Lys Ser Ile Thr Gly Val Gly Leu Val Ser Asp Val
125 130 135

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ttc aat gag tca aag ctt gac caa ctc cct ggt gac atg gca att ggc    483
Phe Asn Glu Ser Lys Leu Asp Gln Leu Pro Gly Asp Met Ala Ile Gly
        140                 145                 150

cac gta agg tac tct act gct ggc tct tct atg tta aaa aat gtt cag    531
His Val Arg Tyr Ser Thr Ala Gly Ser Ser Met Leu Lys Asn Val Gln
    155                 160                 165

cct ttt gtt gct agt tat aaa ttt ggg tca gtt ggt gtt gcc cat aat    579
Pro Phe Val Ala Ser Tyr Lys Phe Gly Ser Val Gly Val Ala His Asn
170                 175                 180                 185

ggt aat tta gtg aat tat aag tta ctg cgt agt gaa cta gag gaa aat    627
Gly Asn Leu Val Asn Tyr Lys Leu Leu Arg Ser Glu Leu Glu Glu Asn
                190                 195                 200

ggg tca att ttt aat aca agt tct gat act gag gtt gta ctt cac ctt    675
Gly Ser Ile Phe Asn Thr Ser Ser Asp Thr Glu Val Val Leu His Leu
            205                 210                 215

att gct ata tct aaa gct agg cca ttt tta ttg agg att gtt gag gct    723
Ile Ala Ile Ser Lys Ala Arg Pro Phe Leu Leu Arg Ile Val Glu Ala
        220                 225                 230

tgt gaa aaa att gaa ggt gct tat tct atg gtg ttt gtt act gag gat    771
Cys Glu Lys Ile Glu Gly Ala Tyr Ser Met Val Phe Val Thr Glu Asp
    235                 240                 245

aag ttg gtt gcc gta agg gat cct cat ggg ttt agg cca ttg gtt atg    819
Lys Leu Val Ala Val Arg Asp Pro His Gly Phe Arg Pro Leu Val Met
250                 255                 260                 265

ggt agg aga agt aat ggt gct gtt gtt ttc gcg tct gag acg tgt gct    867
Gly Arg Arg Ser Asn Gly Ala Val Val Phe Ala Ser Glu Thr Cys Ala
                270                 275                 280

ttg gat ttg att gag gct act tat gag agg gag gtg aat cct ggt gag    915
Leu Asp Leu Ile Glu Ala Thr Tyr Glu Arg Glu Val Asn Pro Gly Glu
            285                 290                 295

gtt gtt gtt gtg gat aaa gat ggg gtt cag tct att tgt ttg atg cct    963
Val Val Val Val Asp Lys Asp Gly Val Gln Ser Ile Cys Leu Met Pro
        300                 305                 310

cat cct gag cgt aaa tct tgt atc ttt gag cat att tac ttt gct ctg   1011
His Pro Glu Arg Lys Ser Cys Ile Phe Glu His Ile Tyr Phe Ala Leu
    315                 320                 325
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cct aat tcg gtc gtg ttt ggg agg tct gtg tac gag tct agg cgt gct 1059
Pro Asn Ser Val Val Phe Gly Arg Ser Val Tyr Glu Ser Arg Arg Ala
330 335 340 345

ttc ggg gag att ctt gct act gaa gct ccc gtg gaa tgt gat gtt gtg 1107
Phe Gly Glu Ile Leu Ala Thr Glu Ala Pro Val Glu Cys Asp Val Val
350 355 360

ata gca gtt cct gac tcg ggt gtc gtg gct gcg ctc ggt tat gct gct 1155
Ile Ala Val Pro Asp Ser Gly Val Val Ala Ala Leu Gly Tyr Ala Ala
365 370 375

aaa gca ggg gta ccg ttt caa caa ggt ttg att agg tcg cat tat gtt 1203
Lys Ala Gly Val Pro Phe Gln Gln Gly Leu Ile Arg Ser His Tyr Val
380 385 390

ggt agg acg ttc atc gag cca tcg cag aag ata agg gat ttc ggg gtg 1251
Gly Arg Thr Phe Ile Glu Pro Ser Gln Lys Ile Arg Asp Phe Gly Val
395 400 405

aag ctt aag ctg tcg ccg gtt agg gcg gtg ttg gag gga aaa aga gtt 1299
Lys Leu Lys Leu Ser Pro Val Arg Ala Val Leu Glu Gly Lys Arg Val
410 415 420 425

gtg gtc gtg gat gat tcg atc gtt aga gga acg acc tcg tcc aag att 1347
Val Val Val Asp Asp Ser Ile Val Arg Gly Thr Thr Ser Ser Lys Ile
430 435 440

gtg agg ctg tta aag gag gcg ggt gcg aaa gag gtt cat atg agg att 1395
Val Arg Leu Leu Lys Glu Ala Gly Ala Lys Glu Val His Met Arg Ile
445 450 455

gca agc cca cca att ata gct tct tgt tat tat gga gtg gat act cct 1443
Ala Ser Pro Pro Ile Ile Ala Ser Cys Tyr Tyr Gly Val Asp Thr Pro
460 465 470

agt tca gat gag ttg ata tca aat agg atg agt gtg gag gag att aag 1491
Ser Ser Asp Glu Leu Ile Ser Asn Arg Met Ser Val Glu Glu Ile Lys
475 480 485

gag ttc att gga tcg gat tcg ctt gct ttt ctg cca atg gat agc ttg 1539
Glu Phe Ile Gly Ser Asp Ser Leu Ala Phe Leu Pro Met Asp Ser Leu
490 495 500 505

aat aag ctc tta ggc aat gat tct aaa agc ttt tgc tat gct tgc ttt 1587
Asn Lys Leu Leu Gly Asn Asp Ser Lys Ser Phe Cys Tyr Ala Cys Phe
510 515 520

tcg ggc aat tac cca gtc gag ccg acg ggt aag gtt aaa agg ata ggg 1635
Ser Gly Asn Tyr Pro Val Glu Pro Thr Gly Lys Val Lys Arg Ile Gly
525 530 535

gat ttc atg gat gat gga tta agt gga gat atg gat tcc att gat ggt 1683
Asp Phe Met Asp Asp Gly Leu Ser Gly Asp Met Asp Ser Ile Asp Gly
540 545 550

gga tgg cta cca gga agt agt agg gtt caa aag act atc ttg aat gaa 1731
Gly Trp Leu Pro Gly Ser Ser Arg Val Gln Lys Thr Ile Leu Asn Glu
555 560 565

gtt aga acc agc taaactttct tttccatgtt tgctttagtt tttgctttgg 1783
Val Arg Thr Ser
570

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Leu Leu Ser Leu Ser Pro Lys Thr His Pro Lys Pro Tyr Arg Thr Leu
35 40 45

Ile Thr Ala Ser Ser Lys Asn Pro Leu Asn Asp Val Ile Ser Phe Lys
50 55 60

Lys Ser Ala Asp Asn Thr Leu Asp Ser Tyr Phe Asp Asp Asp Asp Lys
65 70 75 80

Pro Arg Glu Glu Cys Gly Val Val Gly Ile Tyr Gly Asp Ser Glu Ala
85 90 95

Ser Arg Leu Cys Tyr Leu Ala Leu His Ala Leu Gln His Arg Gly Gln
100 105 110

Glu Gly Ala Gly Ile Val Ala Val Asn Asp Asp Val Leu Lys Ser Ile
        115   120   125

Thr Gly Val Gly Leu Val Ser Asp Val Phe Asn Glu Ser Lys Leu Asp
    130   135   140

Gln Leu Pro Gly Asp Met Ala Ile Gly His Val Arg Tyr Ser Thr Ala
145   150   155   160

Gly Ser Ser Met Leu Lys Asn Val Gln Pro Phe Val Ala Ser Tyr Lys
                165   170   175

Phe Gly Ser Val Gly Val Ala His Asn Gly Asn Leu Val Asn Tyr Lys
            180   185   190

Leu Leu Arg Ser Glu Leu Glu Glu Asn Gly Ser Ile Phe Asn Thr Ser
        195   200   205

Ser Asp Thr Glu Val Val Leu His Leu Ile Ala Ile Ser Lys Ala Arg
    210   215   220

Pro Phe Leu Leu Arg Ile Val Glu Ala Cys Glu Lys Ile Glu Gly Ala
225   230   235   240

Tyr Ser Met Val Phe Val Thr Glu Asp Lys Leu Val Ala Val Arg Asp
                245   250   255

Pro His Gly Phe Arg Pro Leu Val Met Gly Arg Arg Ser Asn Gly Ala
            260   265   270

Val Val Phe Ala Ser Glu Thr Cys Ala Leu Asp Leu Ile Glu Ala Thr
        275   280   285

Tyr Glu Arg Glu Val Asn Pro Gly Glu Val Val Val Val Asp Lys Asp
    290   295   300

Gly Val Gln Ser Ile Cys Leu Met Pro His Pro Glu Arg Lys Ser Cys
305   310   315   320

Ile Phe Glu His Ile Tyr Phe Ala Leu Pro Asn Ser Val Val Phe Gly
                325   330   335

Arg Ser Val Tyr Glu Ser Arg Arg Ala Phe Gly Glu Ile Leu Ala Thr
            340   345   350

Glu Ala Pro Val Glu Cys Asp Val Val Ile Ala Val Pro Asp Ser Gly
        355   360   365

Val Val Ala Ala Leu Gly Tyr Ala Ala Lys Ala Gly Val Pro Phe Gln
370 375 380

Gln Gly Leu Ile Arg Ser His Tyr Val Gly Arg Thr Phe Ile Glu Pro
385 390 395 400

Ser Gln Lys Ile Arg Asp Phe Gly Val Lys Leu Lys Leu Ser Pro Val
405 410 415

Arg Ala Val Leu Glu Gly Lys Arg Val Val Val Asp Asp Ser Ile
420 425 430

Val Arg Gly Thr Thr Ser Ser Lys Ile Val Arg Leu Leu Lys Glu Ala
435 440 445

Gly Ala Lys Glu Val His Met Arg Ile Ala Ser Pro Pro Ile Ile Ala
450 455 460

Ser Cys Tyr Tyr Gly Val Asp Thr Pro Ser Ser Asp Glu Leu Ile Ser
465 470 475 480

Asn Arg Met Ser Val Glu Glu Ile Lys Glu Phe Ile Gly Ser Asp Ser
485 490 495

Leu Ala Phe Leu Pro Met Asp Ser Leu Asn Lys Leu Leu Gly Asn Asp
500 505 510

Ser Lys Ser Phe Cys Tyr Ala Cys Phe Ser Gly Asn Tyr Pro Val Glu
515 520 525

Pro Thr Gly Lys Val Lys Arg Ile Gly Asp Phe Met Asp Asp Gly Leu
530 535 540

Ser Gly Asp Met Asp Ser Ile Asp Gly Gly Trp Leu Pro Gly Ser Ser
545 550 555 560

Arg Val Gln Lys Thr Ile Leu Asn Glu Val Arg Thr Ser
565 570